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The multi-feedstock biorefinery – Assessing the compatibility of alternative feedstocks in a 2G wheat straw biorefinery process

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Abstract

For second-generation (2G) bioethanol refineries, the feedstock supply is one of the important parameters in terms of cost and consistency. Biorefineries are in most cases designed for a specific type of feedstock. For some biorefineries, the use of multiple feedstocks is an option, but how would such feedstocks perform when used in a process designed and optimized for a specific feedstock? There is no “one-size-fits-all” processing package, due to variations in composition and structure of different feedstock types, but due to the size of commercial biorefineries, only minor adjustments of the processing parameters are practically feasible. In this study, 16 alternative feedstocks were characterized and compared to the benchmark feedstock wheat straw under identical processing conditions. The alternative feedstocks studied were as follows: barley straw, rye straw, grass straw, oat straw, Norway spruce sawdust, mixed softwood sawdust, oat wrap, biogas fiber, deep litter, washed deep litter, ryegrass fiber, lucerne fiber, ryegrass chaff, mixed grain chaff, rapeseed press cake, and beer production mash. These biomasses varied in carbohydrate content and accessibility after hydrothermal pretreatment. Applying a hydrothermal pretreatment under identical conditions, the subsequent enzymatic convertibility of these biomasses ranged from 0.5% to complete conversion based on their glucan content. Water retention value was determined and correlated with enzymatic convertibility, which provided a simple method for indirect measurement of biomass recalcitrance. Ethanol potentials were estimated based on carbohydrate release from enzymatic hydrolysis, and yeast toxicity test was performed on liquid fractions from hydrothermal pretreatment. Furthermore, a number of key processing indicators, including market price, logistics and availability, were taken into consideration based on a proposed full-scale 2G ethanol plant in Denmark. The overall results show that while some feedstocks had inferior performance compared to wheat straw, identical or even superior performance was observed from barley, oat, and ryegrass feedstocks.

KEYWORDS

biorefinery, comprehensive microarray polymer profiling, enzymatic hydrolysis, ethanol potential, hydrothermal pretreatment, lignocellulose, water retention value

1 | INTRODUCTION

Some agricultural areas are dominated heavily by one crop type, such as maize in the corn belt of the United States. In such a scenario, the second-generation (2G) bioethanol refineries will be optimized specifically for these crops. In other areas, adjacent fields vary greatly in crop types, as, for example, in most of Europe. This imposes challenges for proposed 2G ethanol refineries, since the unit operations vary for different agricultural residues, as there is no “one-size-fits-all” for pretreatment and enzymatic deconstruction of lignocellulosic plant cell walls. Another issue in lignocellulosic biomass processing is the low biomass density, which makes long-distance transportation uneconomical, while the plants need large biomass inflows due to economy-of-scale in the processing. Therefore, to ensure a sufficient and consistent feedstock supply, plant owners are compelled to include as many local biomass types in their production as possible. Furthermore, increasing the feedstock diversity will create value to several stakeholders in the supply chain, by expanding the feedstock market to enter the large-scale 2G bioethanol refinery. Also, this will increase feedstock resource security as well as competition at the supplier side lowering the feedstock costs.

Pretreatment for lignocellulosic biomass has been studied extensively in the past decades, and the optimal conditions differ between biomass types (Garlock et al., 2011; Thomsen, Londoño, Schmidt, & Kádár, 2015). The biorefinery may operate in campaigns with optimized conditions for each biomass. However, this imposes logistic- and storage-wise complications. Alternatively, the biorefinery could operate continuous processing settings optimized for the most common biomass and only use other biomass feedstocks, which are compatible under these settings.

Hydrothermal pretreatment is used at industrial scale, often without addition of chemicals. The method partially hydrolyzes hemicelluloses and relocates the lignin (Hansen, Kristensen, Felby, & Jørgensen, 2011; Jørgensen, Kristensen, & Felby, 2007; Thomsen et al., 2015). Many research articles and industrial reports have addressed hydrothermal pretreatment as an effective pretreatment strategy for wheat straw biomass (Larsen, Petersen, Thirup, Li, & Iversen, 2008; Mosier et al., 2005; Petersen, Larsen, & Thomsen, 2009; Thomsen, Thygesen, & Thomsen, 2008). The reported optimized conditions differ slightly between different studies, due to differences in pretreatment unit and scale, but several authors have reported 190°C for 10 min as an optimum for wheat straw (Ambye-Jensen, Thomsen, Kádár, & Meyer, 2013; Ertas, Han, Jameel, & Chang, 2014; Zhang et al., 2014). Using existing pretreatment technology and infrastructure optimized for wheat straw biomass, it could be economically efficient to add biomass feedstock diversity to the biorefinery on a local/

regional supply basis. However, scientific studies assessing compatibility alternative feedstocks to the processing conditions of the most abundant local biomass type have not been undertaken so far.

In this study, in the context of 2G bioethanol industrialization, we aim at understanding a range of alternative feedstocks from a chemical/biological perspective, assessing compatibility to a wheat straw-based biorefinery. Seventeen types of biomass were included, ranging from agricultural residues, woody biomasses, to processed fibers. Biomass samples were chemically characterized, and the accessibility of hemicellulose polysaccharides in each biomass type was examined using comprehensive microarray polymer profiling (CoMPP). The influence of physical/chemical pretreatment on the level of biomass recalcitrance involves a number of highly complex factors. Water retention value (WRV) has been reported as a simple predictive indicator for recalcitrance (Weiss, Thygesen, Felby, Roslander, & Gourlay, 2017), and in this work, it is studied to which extent this correlation persists over a broad spectrum of biomasses. WRV measurement may be a tool for evaluation of alternative feedstocks. The ethanol potential was calculated on grounds of mono- and oligosaccharides recovered from pretreatment and the yield from enzymatic hydrolysis. Furthermore, the fermentability of the pretreatment liquors was tested by a toxicity test for yeast viability in liquid fractions from hydrothermal pretreatment. Last but not the least, a number of key processing indicators were taken into consideration based on a proposed full-scale 2G ethanol plant in Denmark.

2 | MATERIAL AND METHODS

2.1 | Biomass materials

The biomasses were produced and collected in the catchment area of Maabjerg Energy Center, Holstebro, Denmark, and were all perceived as potential resources for a prospected 2G ethanol plant. The only exceptions were ryegrass fiber (RF) and lucerne fiber (LF) residues from a mechanical protein extraction unit placed at Aarhus University, Foulum, Denmark (Hermansen et al., 2017). The washed deep litter (WDL) is from the same source as the deep litter (DL), but were washed by suspending 30 g total solids (TS) DL for 3 times in a total of 750 ml water. The biomasses were produced and collected during 2016 and 2017. The dry biomasses were stored dry and cool (5°C), while moist biomasses (<85% TS) were stored at −20°C. The dry straw materials, namely wheat straw (WS), barley straw (BS), rye straw (RS), ryegrass straw (GS, harvested for grass seeds at maturity) and oat straw (OS), were knife milled to pass a 1.5-cm sieve. The moist biomasses (<85% TS) with long fibers, including oat wrapped silage (OW),

deep litter (DL), washed deep litter (WDL), ryegrass fiber (RF), and lucerne fiber (LF), were size reduced with scissors to <1 cm. The remaining biomasses, Norway spruce sawdust (NSS), mixed softwood sawdust (MSS), biogas fiber (BF-fibers separated by centrifugation from anaerobically digested manure at industrial scale), ryegrass chaff (RC), mixed grain chaff (MGC), rapeseed press cake (RPC), and beer production mash (BPM), were not further size reduced since they were already able to pass a 1.5-cm sieve.

2.2 | Hydrothermal pretreatment of biomasses

The pretreatments were performed in a Dionex™ ASE™ 350 Accelerated Solvent Extractor (CA, USA), as reported previously (Wolfrum, Ness, Nagle, Peterson, & Scarlata, 2013). All biomasses were pretreated in conditions optimized for WS; that is, 15 g of TS was added to a 100 ml extraction cell, the cells were filled up with MQ-water and heated to 190°C (heating time 9 min), and the cells were kept at this temperature for 10 min and, hereafter, the cells were emptied into a collection vial (rinse volume 10% of the pretreatment volume, purge time 100 s). The liquid fraction was collected separately and kept at −20°C until further use. The solid fraction was washed on the ASE (2 cycles, 40°C, 1-min static time, 10% rinse volume) and stored at −20°C until further use.

2.3 | Total solids and ash content determination

Total solids % and ash content were determined for all raw and pretreated material in triplicates by use of the current standard NREL method (Sluiter et al., 2008).

2.4 | Carbohydrate analysis

Biomass samples were dried in freeze dryer for 24 hr and then ball-milled for 3 min using a TissueLyser (QIAGEN, Copenhagen, Denmark), prior to analysis. Strong acid hydrolysis was performed as a downscaled version of the standard method (Sluiter, Ruiz, Scarlata, Sluiter, & Templeton, 2010). 4 mg of each biomass was carefully weighed into a 1.5-ml glass vial (Webseal insert, 1.5 ml, U-base; Thermo Scientific, Germany). Five replicates were made for each biomass type. 40 µl sulfuric acid (72% w/w) was added to each vial, followed by vortex mixing. Glass vials were placed in a deep 96-well plate after mixing. The deep well plate was then transferred to a preheated plate incubator, at 30°C, 900 rpm for 1 hr. Glass vials were taken to vortex after about 30 min. 1.1 ml MilliQ water was added to each glass vial after incubation. The glass vials were covered by

a Teflon film with a little hole on each vial and then loosely wrapped with aluminum foil. The plate was autoclaved at 121°C, for 1 hr. 300 µl liquid from each vial was transferred into a 2-ml 96-well plate, followed by pH neutralization using concentrated sodium carbonate solution (140 g/L). Samples were filtered using a 96-well plate filter (0.45 µm) for monosaccharide determination on HPLC. Data are summarized in Table 1. Lignin content and the contents of various extractives were not determined in this study.

2.5 | Comprehensive microarray polymer profiling

Dried, finely ground, aliquots of each sample (10 mg) were weighed in triplicate, and 300 µl of 50 mM trans-1,2-diaminocyclohexane-*N,N,N',N'*-tetraacetic acid (CDTA) pH 7.5 was added in order to extract pectic polysaccharides. The samples were gently shaken for 2 hr at room temperature before centrifugation at 2,500 g for 10 min. The supernatant, which contains the solubilized pectin fraction, was removed and stored for later use. To the pellet, 300 µl of 4 M NaOH with 0.1% (v/v) NaBH₄ was added to extract hemicellulose polysaccharides. Again, the samples were shaken for 2 hr and centrifuged for 10 min, and the supernatants containing the solubilized cell wall polymers were collected. The supernatant from both extractions for all samples was diluted 2-, 10-, 50-, and 250-fold in phosphate-buffered saline (PBS) buffer (140 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.7 mM KH₂PO₄, pH 7.5) and printed onto nitrocellulose membranes, in triplicate, using an Arrayjet Sprint Inkjet Microarrayer (Arrayjet, UK). Arrays were cutout, blocked for 1 hr with 5% fat-free milk protein in PBS, and probed with primary monoclonal antibodies (mAbs) or carbohydrate-binding molecules (CBMs) for 2 hr at room temperature, as described by Willats, Marcus, and Knox (1998) and McCartney, Marcus, and Knox (2005). After washing repeatedly in PBS, the arrays were probed with a secondary antibody conjugated to alkaline phosphatase for a further 2 hr. The arrays were washed again in PBS and in distilled H₂O prior to development with BCIP/NBT (5-bromo-4-chloro-3'-indolylphosphate/nitroblue tetrazolium chloride). Once dry, the arrays were scanned using a flatbed scanner at 1,200 dpi and converted to a negative image, 16-bit, gray-scale TIFFs, which were uploaded to the IMA GENE 6.0 microarray analysis software. Processed and analyzed data were converted to a heatmap (Moller et al., 2007). The mAbs used in this study are listed in Table 2. The heatmap digest is presented in Figure 1.

2.6 | Enzymatic hydrolysis

Enzymatic hydrolysis of the pretreated biomasses was carried out at 15% TS, 1 g TS per replicate (3 replicates) at

TABLE 1 Proportions of main carbohydrate components and ash content in 17 studied feedstocks with and without pretreatment on the basis of dry weight in percentage (g/100 g TS)

Category	Biomass	g/100 g TS					
		Glucan	Xylan	Arabinan	Galactan	Mannan	Ash
Straw	WS	38.2 (1.5)	26.6 (1.1)	1.3 (0.1)	n.d.	n.d.	3.4 (0.0)
	BS	34.5 (2.4)	22.1 (1.1)	1.0 (0.0)	n.d.	n.d.	4.7 (0.0)
	RS	38.8 (2.0)	21.7 (1.3)	0.9 (0.1)	n.d.	n.d.	2.2 (0.0)
	GS	31.9 (2.1)	20.7 (0.5)	1.4 (0.1)	n.d.	n.d.	5.7 (0.0)
	OS	39.8 (1.4)	21.6 (1.6)	1.0 (0.0)	n.d.	n.d.	3.9 (0.0)
Softwood	NSS	40.7 (1.0)	4.8 (0.4)	1.0 (0.1)	2.0 (0.1)	11.1 (0.6)	0.3 (0.0)
	MSS	42.7 (1.0)	4.7 (0.4)	0.9 (0.1)	2.0 (0.1)	11.1 (0.7)	0.4 (0.0)
Processed fiber	OW	35.5 (1.5)	17.8 (1.1)	0.9 (0.0)	n.d.	n.d.	8.1 (0.4)
	BF	10.1 (0.9)	2.7 (0.2)	3.3 (0.1)	n.d.	n.d.	29.7 (0.5)
	DL	26.4 (0.7)	13.5 (0.9)	1.0 (0.0)	n.d.	n.d.	15.1 (2.0)
	WDL	37.8 (2.4)	22.5 (1.1)	1.2 (0.1)	n.d.	n.d.	9.1 (1.2)
	RF	24.8 (1.5)	15.8 (0.5)	2.1 (0.2)	n.d.	n.d.	6.5 (0.4)
	LF	30.7 (2.9)	5.7 (0.9)	0.6 (0.1)	0.3	n.d.	4.8 (0.3)
	RC	35.8 (2.2)	18.4 (0.2)	1.2 (0.1)	n.d.	n.d.	6.0 (0.1)
	MGC	40.3 (1.0)	6.8 (0.6)	1.9 (0.1)	n.d.	n.d.	13.7 (1.4)
	RPC	12.0 (2.3)	1.5 (0.2)	4.7 (0.1)	2.5 (0.1)	0.5 (0.0)	7.4 (0.4)
	BPM	28.0 (2.0)	6.4 (0.5)	7.0 (0.7)	0.7 (0.2)	0.2 (0.0)	3.4 (0.1)
Straw (Pretreated)	WS-P	39.6 (2.2)	21.5 (1.2)	1.4 (0.1)	n.d.	n.d.	0.0 (0.0)
	BS-P	42.6 (2.4)	20.4 (1.5)	1.3 (0.1)	n.d.	n.d.	2.5 (0.0)
	RS-P	44.6 (0.9)	17.4 (1.0)	1.1 (0.0)	n.d.	n.d.	0.6 (0.0)
	GS-P	31.5 (2.7)	14.8 (1.2)	1.2 (0.1)	n.d.	n.d.	2.7 (0.0)
	OS-P	42.6 (1.3)	18.8 (1.2)	1.5 (0.4)	n.d.	n.d.	2.0 (0.0)
Softwood (Pretreated)	NSS-P	53.6 (1.1)	3.3 (0.2)	n.d.	0.4 (0.1)	3.9 (0.1)	0.2 (0.1)
	MSS-P	53.5 (2.9)	2.8 (0.2)	n.d.	0.4 (0.0)	3.0 (0.2)	0.3 (0.1)
Processed fiber (Pretreated)	OW-P	37.4 (1.8)	17.3 (1.6)	1.3 (0.1)	n.d.	n.d.	3.5 (0.6)
	BF-P	18.0 (1.1)	12.0 (0.6)	3.7 (0.3)	n.d.	n.d.	32.0 (1.0)
	DL-P	29.0 (1.0)	12.8 (1.1)	1.0 (0.0)	n.d.	n.d.	10.6 (2.6)
	WDL-P	36.5 (0.9)	14.8 (1.2)	1.2 (0.0)	n.d.	n.d.	5.5 (0.2)
	RF-P	38.0 (0.7)	13.2 (0.8)	0.7 (0.0)	n.d.	n.d.	7.6 (1.2)
	LF-P	41.0 (1.3)	5.6 (0.2)	0.2 (0.0)	n.d.	n.d.	5.3 (0.9)
	RC-P	22.0 (1.2)	13.4 (2.3)	1.1 (0.2)	n.d.	n.d.	4.1 (0.2)
	MGC-P	19.7 (2.4)	9.6 (0.8)	1.1 (0.1)	n.d.	n.d.	18.5 (1.3)
	RPC-P	14.5 (1.5)	1.4 (0.2)	1.0 (0.1)	1.1 (0.1)	0.8 (0.1)	6.5 (1.5)
	BPM-P	28.8 (0.8)	4.3 (0.1)	1.5 (0.1)	0.6 (0.2)	0.4 (0.1)	3.1 (0.3)

Note. Values presented are the average numbers of five replicates. Values presented in parentheses are standard deviation (*SD*) numbers ($n = 5$). n.d.: not detected.

50°C for 72 hr in a free fall tumbler (10 rpm) previously described (Zhang, Fredriksson, Mravec, & Felby, 2017). The hydrolysis was performed at pH 5.0 in 50 mM citrate buffer. The enzyme loading was held constant (independent of cellulose content) at 0.024 g enzyme solution per g TS. The enzyme blend was the cellulase blend Cellic® CTec3 kindly provided by Novozymes (Bagsværd, Denmark). Cellulase activity in filter paper unit (FPU) (g/DM) was

estimated based on a standard method to 73 (Ghose, 1987). However, it should be emphasized that FPU/g does not account for the full spectrum of enzyme activities in present-day enzyme cocktails and that relatively bad reproducibility is associated with the method. The reaction was terminated by heating the tubes to 95°C for 15 min, hereafter subsamples were spun and sugars were analyzed by HPLC.

TABLE 2 List of mAbs in this study

	Specificity	Reference
LM5	(1 → 4)-β-D-galactan	Jones, Seymour, and Knox (1997)
LM6	(1 → 5)-α-L-arabinan	Willats et al. (1998)
LM10	(1 → 4)-β-D-xylan	McCartney et al. (2005)
LM11	(1 → 4)-β-D-xylan/ arabinoxylan	McCartney et al. (2005)
LM13	Linearized (1 → 5)-α-L- arabinan	Moller et al. (2007)
LM21	(1 → 4)-β-D-mannan	Marcus et al. (2010)
LM22	(1 → 4)-β-D-mannan/ galactomannan	Marcus et al. (2010)
LM25	Xyloglucan	Pedersen et al. (2012)
BS-400-3	(1 → 3)(1 → 4)-β-D- glucan	Meikle, Hoogenraad, Bonig, Clarke, and Stone (1994)

2.7 | Water retention value determination

Water retention value measured the mass of water retained by biomass after centrifugation at 3,000 *g* for 15 min. The analyses were performed on 482 mg TS pretreated biomass added as wet “never-dried” material, as previously described by Weiss et al. (2017).

2.8 | Monosaccharides, toxicants, and ethanol analyses

Monosaccharides (D-glucose, D-xylose, and L-arabinose) released from enzymatic hydrolysis, toxicants (furfural, 5-hydroxymethyl furfural, acetate) generated from pretreatment, and ethanol produced from fermentation were measured on an Ultimate 3000 HPLC system (Dionex, CA, USA) fitted with a Phenomenex Resex ROA column at 80°C with 5 mM H₂SO₄ as eluent at a flow rate of 0.6 ml/min. The samples were diluted in eluent, filtered through a 0.45-μm nylon filter before injection.

Monosaccharides from carbohydrate analysis (L-arabinose, D-galactose, D-glucose, D-mannose, and D-xylose) were determined using an ICS 5000 system (Dionex). The separation was performed in a Dionex CarboPac PA1 column at 30°C with a flow rate of 1 ml/min of MQ-water. Detector sensitivity was optimized by post-column addition of 200 mM NaOH at a flow rate of 0.5 ml/min. The column was cleaned after each sample with 0.25 M NaOH for 5 min and reconditioned by MQ-water for 5 min.

2.9 | Toxicity test

One millilitre of *Saccharomyces cerevisiae* ethanol red (Lesaffre Advanced Fermentations), grown in YPD for

15 hr at 37°C and 200 rpm was diluted 1,000 times, plated in a solid YPD-agar plate, and incubated at 37°C for 48 hr, prior to storage at 5°C. From each plate, one colony was added to 100 ml of YPD in a 250-ml shake flask to prepare the pre-culture, which was grown at 30°C, 120 rpm for 15 hr. After solid fraction removal by centrifugation, 40 ml liquid fraction from each pretreated mixture was transferred to a 50-ml Falcon tube, followed by addition of 22 g/L of glucose. All Falcon tubes were inoculated from the pre-culture to an OD₆₀₀ of 1.5. The volume of each tube was adjusted to 50 ml with deionized water. Each mixture was split and dispensed into two 250-ml shake flasks (25 ml volume in each shake flask). The shake flasks were incubated in an orbital shaker at 30°C, 120 rpm for 9–15 hr. 1 ml hourly sample from each shake flask monitored the optical density during fermentation. A 1 ml sample was taken from each shake flask for every 2 hr, filtrated via 0.20-μm cellulose acetate filter, and stored at –20°C for HPLC analysis.

2.10 | Elemental analysis

The raw material samples were digested using HNO₃, H₂O₂, and HF, in and in a pressurized microwave oven, and multi-elemental analyses were conducted using inductively coupled plasma–optical emission spectroscopy (ICP-OES) through a previously described method (Cabrera, Cabrera, Jensen, & Felby, 2016). Data are presented in Supporting Information Table S1.

2.11 | Ethanol potential

The ethanol potentials were calculated from three components assuming full conversion and hydrolytic gain. (a) The free sugars present in the pretreatment liquor after pretreatment; (b) the sugars from oligomeric soluble carbohydrates present in the pretreatment liquor after pretreatment (conversion factor is 1.14 g pentose sugar/g pentose oligomer and 1.11 g hexose sugar/g hexose oligomer); (c) the free sugars after enzymatic hydrolysis of the solid fraction. After a mass balance, these three components were calculated into ethanol yield per gram raw material with sugar to ethanol stoichiometric conversion factors (0.51 g ethanol/g sugar). The ethanol potentials were based on specific sugars fermentable by industrial yeast strains. *S. cerevisiae* can naturally convert all the hexoses: glucose, mannose, and galactose, and genetically engineered strains can efficiently ferment the pentose xylose (Tomás-Pejó, Bonander, & Olsson, 2014); thus, these sugars are included in the ethanol potential. Industrial strains fermenting arabinose have not yet been reported; thus, arabinose was not accounted for calculating the ethanol potentials.

2.12 | Statistics

The open-source software “R” was used for statistical computing. The analysis of variance (AOV)-function was used for the one-way ANOVAs. Tukey multiple comparisons of means (95% family-wise confidence level) were performed based on the Studentized range statistic and Tukey's “honest significant difference” method (the Tukey HSD-function in R).

2.13 | Key processing indicators

Price estimate, logistics, and availability were assessed by expert judgments by the local stakeholder MEC, who are presently sources of biomass for full-scale biogas and CHP plants. The indicators is presented in a 6-step scale from --- to +++, +++ being most optimal. The ethanol potential and fermentability are based on laboratory data scaled similarly.

3 | RESULTS

3.1 | Alternative feedstock characterization

The characterization of the studied alternative feedstocks included cell wall structural carbohydrate analysis (Table 1) and accessibility of hemicelluloses using CoMPP method (Figure 1). The mAbs used in this study were listed in Table 2.

3.2 | Straw type feedstocks

Wheat straw had a slight increase in glucan content by 1.4 g/(100 g TS) and a noticeable drop in xylan content by 5.1 g/(100 g TS) after the pretreatment (Table 1). The other straw feedstocks, namely, BS, RS, GS, OS, varied in glucan content change introduced by pretreatment (Table 1). BS, RS, and OS had elevated glucan contents by 8.1, 5.8, and 2.8 g/(100 g TS), respectively; and GS remained at the same level after pretreatment (Table 1). Meanwhile, xylan contents of all straw types dropped in a range from 1.7 to 5.9 g/(100 g TS) (Table 1). The ash contents of straw type feedstocks varied from 2.2 to 5.7 g/(100 g TS), and the ash contents contributed to no more than 3 g/(100 g TS) after pretreatment (Table 1).

In CoMPP results, WS, BS, and RS biomasses showed similar signal intensities to the mAbs LM 10 and LM 11 (Figure 1). However, xylan backbone-specific mAb LM 10 showed significantly lower signal intensities to GS and OS, 50% and 47% lower compared to WS (Figure 1). After pretreatment, signal intensities of LM 10 remained at the same level for WS, BS, and RS, but a drastic increase was observed in GS and OS, by 67% and 42%, respectively

(Figure 1). Signal intensities of xylan/arabinoxylan-specific mAb LM 11 in all samples dropped after pretreatment, of which RS showed the largest decline by 33% (Figure 1). The signal intensities given by mixed linkage glucan (MLG)-specific mAb BS-400-3 increased in all straw feedstocks after pretreatment (Figure 1).

Overall, straw type feedstocks had relatively similar structural carbohydrates in quantity, but rather different accessibility to the mAbs. Hydrothermal pretreatment resulted in varied structural carbohydrate changes in quantity, but to a large extent eliminated the different accessibility of the xylan backbone. WS, as the benchmark feedstock, will be compared to the other feedstocks in the following results section.

3.3 | Softwood feedstocks

Norway spruce sawdust had three major structural carbohydrates, namely, glucan (40.7 g/100 g TS), xylan (4.8 g/100 g TS), and mannan (11.1 g/100 g TS) (Table 1). Ash content was negligible. Pretreatment increased its glucan content by 12.9 g/100 g TS and resulted in 1.5 g/(100 g TS) and 7.2 g/(100 g TS) drop for xylan and mannan, respectively (Table 1). The MSS had an identical carbohydrate profile to NSS regardless of pretreatment (Table 1).

The mAbs LM 10 and LM 11 signals emerged after pretreatment for both biomasses (Figure 1), which indicates slightly improved accessibility to xylans. Meanwhile, the signals from mAbs LM 21 and LM 22 disappeared after pretreatment for both biomasses (Figure 1), which suggests that the remaining mannan fraction was shielded.

Compared to WS, the softwood feedstocks had similar glucan content and, as expected, a very different hemicellulose polysaccharides profile.

3.4 | Processed fiber feedstocks

There are 10 different biomasses in this category. OW, WDL, and RC had the most identical carbohydrate profiles as WS. However, the ash contents of these three feedstocks (OW 8.1, WDL 9.1, and RC 6.0 g/100 g TS) were all higher than WS (3.4 g/100 g TS) (Table 1). Besides significant drop in ash content, these three feedstocks behaved very differently during hydrothermal pretreatment. Pretreated OW (OW-P) showed a slight increase in glucan content by 1.9 g/(100 g TS) and a marginal decrease in xylan content (Table 1). Pretreated WDL (WDL-P) retained the same level of glucan, but a sharp drop in xylan content by 7.7 g/(100 g TS). Pretreated RC (RC-P) contained much lower glucan and xylans compared to RC, most likely due to the free sugars removed by washing, and starch (Figure 2a). To highlight from the CoMPP results, hydrothermal pretreatment strongly improved accessibility of

		(1→4)-β-d-xylofuran/arabinofuran (mAb LM11)	(1→4)-β-d-xylofuran (mAb LM10)	Xyloglucan (mAb LM25)	(1→3)(1→4)-β-d-glucan (mAb BS-400-3)	(1→4)-β-d-mannan/galactomannan (mAb LM22)	(1→4)-β-d-mannan (mAb LM21)	Linearised (1→5)-α-L-arabinan (mAb LM13)	(1→5)-α-L-arabinan (mAb LM6)	(1→4)-β-d-galactan (mAb LM5)	
Category	Biomass										
Straw	WS	4	0	0	6	0	32	24	48	95	
	BS	0	0	0	0	0	41	22	43	96	
	RS	0	0	0	0	0	33	21	43	95	
	GS	4	0	0	0	0	41	18	24	82	
	OS	5	0	0	0	0	27	19	31	88	
Softwood	NSS	0	0	0	13	14	0	7	0	0	
	MSS	0	0	0	14	19	5	10	0	0	
Processed fiber	OW	0	0	0	0	0	37	18	30	80	
	BF	0	0	0	0	0	31	21	27	64	
	DL	9	0	0	0	0	30	28	38	77	
	WDL	8	0	0	0	0	32	26	40	77	
	RF	8	0	0	0	0	42	25	28	82	
	LF	11	0	0	0	0	30	39	23	56	
	RC	4	0	0	0	0	43	5	13	61	
	MGC	3	0	0	0	0	17	11	4	35	
	RPC	40	0	0	0	0	0	87	0	0	
	BPM	58	14	11	0	0	0	34	0	18	
	Straw	WS-P	7	0	0	0	0	43	31	47	75
		BS-P	4	0	0	0	0	57	27	49	77
		RS-P	6	0	0	0	0	38	27	43	64
		GS-P	8	0	0	0	0	48	28	40	70
		OS-P	6	0	0	0	0	36	26	44	72
Softwood	NSS-P	0	0	0	2	2	0	11	6	8	
	MSS-P	0	0	0	0	0	0	12	7	6	
Processed fiber	OW-P	2	0	0	0	0	36	23	39	62	
	BF-P	4	0	0	0	0	30	25	30	58	
	DL-P	8	0	0	0	0	40	30	40	58	
	WDL-P	7	0	0	0	0	46	30	37	52	
	RF-P	9	0	0	0	0	51	29	35	48	
	LF-P	12	0	0	0	0	36	39	28	37	
	RC-P	8	0	0	0	0	49	28	39	58	
	MGC-P	7	0	0	0	3	35	27	33	52	
	RPC-P	22	0	0	0	0	0	76	0	0	
	BPM-P	7	0	0	0	0	17	24	19	19	

FIGURE 1 Heatmap of comprehensive microarray polymer profiling (CoMPP) analysis of 17 studied feedstocks with and without pretreatment in NaOH extraction. The numbers in the heatmap indicate the signal intensity (0 → 100, signals from low to high). The specificities of mAbs are listed in Table 2

hemicelluloses in RC, indicated by a fivefold xyloglucan mAb LM 25 signal intensity and a twofold LM 10 signal intensity after the pretreatment (Figure 1). Similar to RC, MGC showed significantly higher signal intensities toward mAbs LM 25 and LM 10, as well as BS-400-3 and LM 11, after pretreatment (Figure 1).

Ryegrass fiber and LF varied largely in their principal carbohydrates. Glucan contents increased by 13.2 g/100 g and 10.3 g/100 g for RF and LF, respectively (Table 1).

Xylan content dropped slightly for RF and remained the same for LF after pretreatment (Table 1). The ash contents of RF and LF increased slightly by 1.2 and 0.5 g/(100 g TS), respectively, after pretreatment (Table 1). The CoMPP results showed significantly decreased LM 11 signal intensities for RF (40%) and LF (33%) (Figure 1).

Beer production mash had a low-end xylan content at 6.4 g/(100 g TS), but the highest arabinan content at 7.0 g/(100 g TS) compared to all the other biomasses (Table 1).

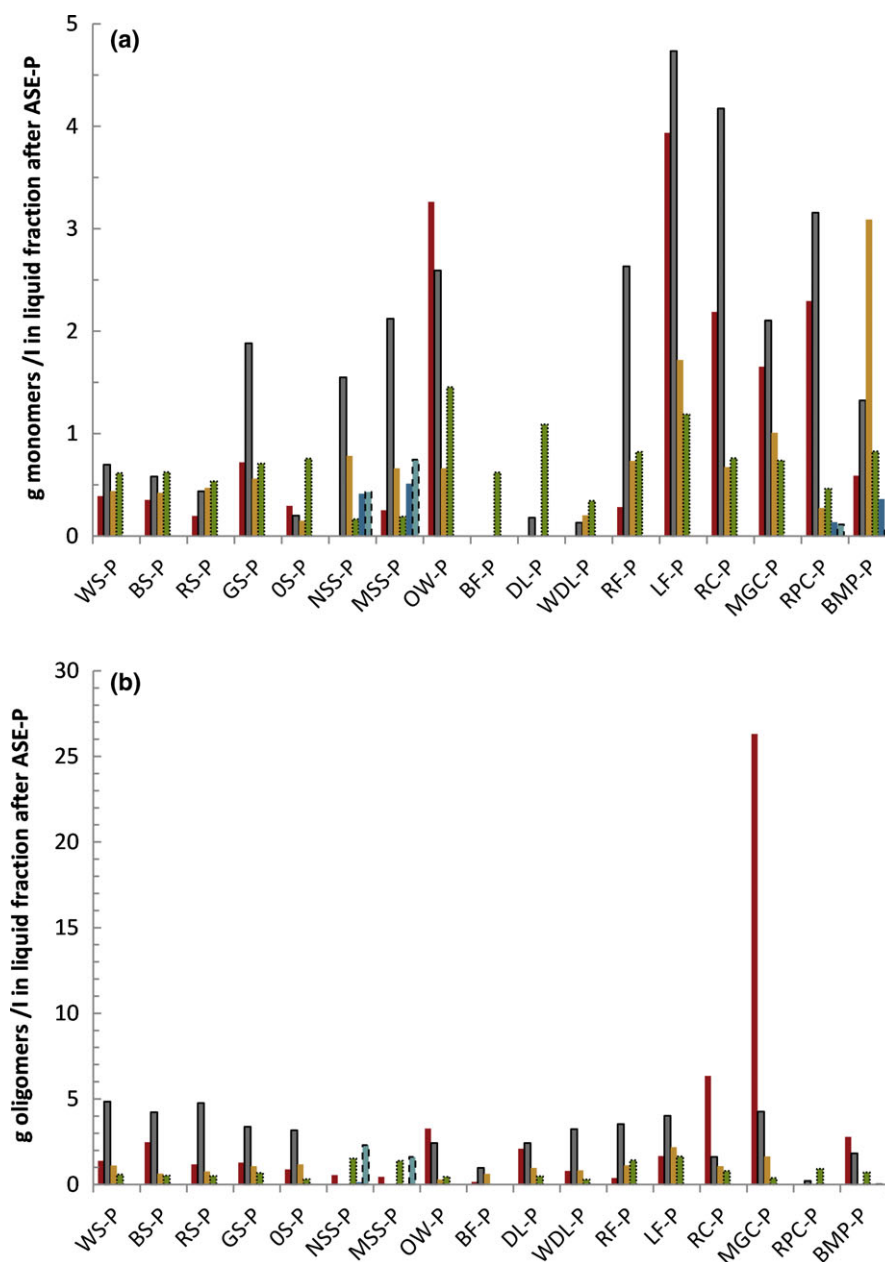


FIGURE 2 Carbohydrates in the liquid fraction after ASE-P. (a) Monomers. (b) Oligomers. Red bars: glucose/glucan. Gray bars with solid outline: xylose/xylan. Yellow bars: arabinose/arabinan. Green bars with dotted outline: acetate. Blue bars: galactose/galactan. Light blue bars with dashed outline: mannose/mannan.

The glucan content remained at same level after pretreatment, but the arabinan content decreased dramatically down to 1.5 g/(100 g TS) (Table 1). MLG and xylan were more accessible after pretreatment, suggested by appearance of BS-400-3 and LM 10 signals (Figure 1).

Rapeseed press cake had a relatively low carbohydrate profile, with 12.0 g/(100 g TS) glucan, 4.7 g/(100 g TS) arabinan, and small amount of xylan (1.5 g/(100 g TS)), galactan (2.5 g/100 g TS), and mannan (0.5 g/100 g TS) (Table 1). Arabinan content dropped sharply after the pretreatment, and the ash content remained at the same level (Table 1). The mAb LM 25 gave strong signal intensity toward RPC and RPC-P, which suggests abundant presence of xyloglucan in RPC (Figure 1). Galactan-specific mAb LM 5 showed nearly half-size signal intensity after

pretreatment (Figure 1), which is consistent to carbohydrate analysis result.

The processed fiber feedstocks demonstrated large variations in carbohydrate content and accessibility; however, similarity in carbohydrate profile was observed in OW, WDL, and RC, when compared to WS. Responses of these processed fiber feedstocks to hydrothermal pretreatment varied to large extent and differed from WS.

3.5 | Enzymatic convertibility and correlations with water retention value

The enzymatic convertibility of all 17 feedstocks varied from complete conversion of pretreated RF (RF-P) to almost no conversion of pretreated BF (BF-P) based on

their theoretical glucan contents (Figure 3). By category, straw type feedstocks showed large variations in their enzymatic convertibility, despite similarities in their carbohydrate profiles and hemicellulose accessibility after pretreatment. The pretreated RS (RS-P) with the highest glucan content resulted in lowest glucan conversion (34%), and pretreated GS (GS-P) gave the highest glucan yield at 95% with the lowest glucan content (Figure 3). Poor enzymatic hydrolysis performance was observed in both softwood feedstocks, which was in line with the CoMPP results, that is, low accessibility to mannan and xylan (Figures 1 and 3). As expected, the processed fiber feedstocks exhibited large variations in their glucan conversion yields, including the best performer RF-P (104%—within expected uncertainties) and the worst performer BF-P (0.5%) among all 17 studied feedstocks.

Water retention value was examined in correlation with glucan conversion yield (Figure 4a,b). Glucan yield was presented as converted g glucan/(g TS) in Figure 4a and as converted g glucan/(g theoretical glucan) in Figure 4b. The regression lines fitted better on the basis of TS ($R^2 = 0.5777$) than on the basis of theoretical glucan ($R^2 = 0.3514$) (Figure 4a,b). In this study, the glucan content of biomasses varied in a broad range (14.5–53.6 g/100 g TS) as part of the biomass TS; therefore, the regression line of glucan yield and WRV correlation fitted better, when taking the whole biomass weight into account. Pretreated NSS (NSS-P), pretreated MSS (MSS-P), pretreated DL (DL-P), pretreated MGC (MGC-P), pretreated RPC (RPC-P), and pretreated BMP (BPM-P) showed lower WRVs, which also correlated with lower glucan conversion yields on TS basis, when compared to pretreated WS (WS-P) (Figure 4a). The biomasses with higher WRVs gave similar or higher glucan conversion yields, compared to WS-P, with only one exception, pretreated RS (RS-P) (Figure 4a).

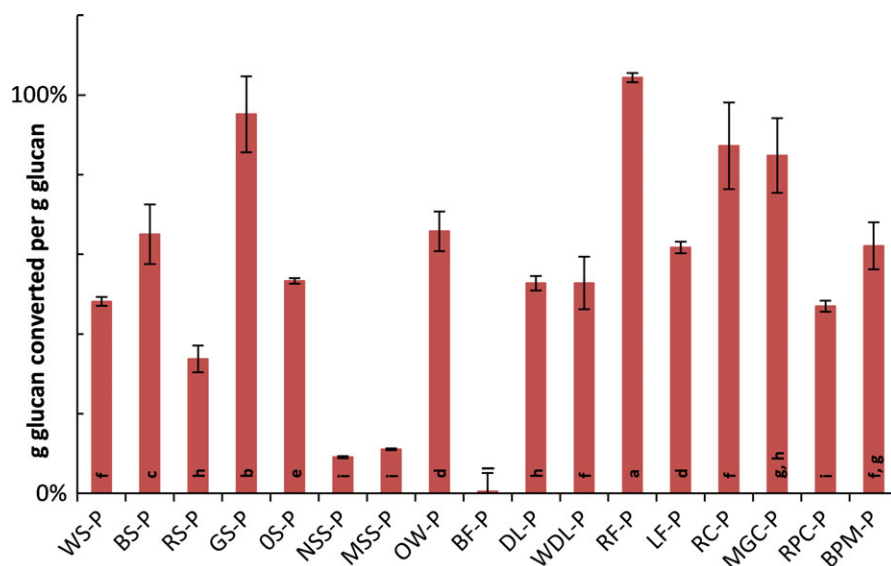


FIGURE 3 Glucan conversion of 17 pretreated alternative feedstocks on the basis of theoretical glucan content of each biomass. Error bars are standard deviation (SD) numbers ($n = 3$), and small letters indicate significant difference

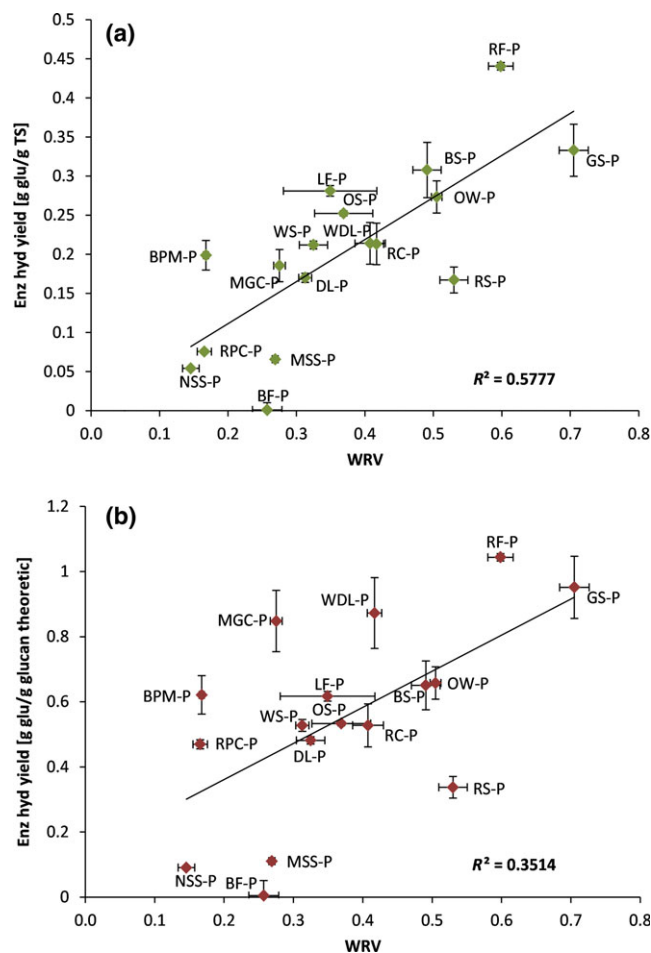


FIGURE 4 Correlation of enzymatic hydrolysis yield and water retention values (WRVs) of 17 pretreated alternative feedstocks. (a): Enzymatic hydrolysis yield is presented in g glucan/(g TS). (b): Enzymatic hydrolysis yield is presented in g glucan/(g theoretical glucan)

3.6 | Ethanol potential and toxicity test

The estimation of ethanol potential yield included carbohydrates released from hydrothermal pretreatment, either as monomers or as oligomers, and monomeric sugars generated during enzymatic hydrolysis (Table 3). As the benchmark biomass, WS gave an ethanol potential at 17.6 g/(100 g TS) (Table 3). BS, GS, OW, RF, LF, and MGC showed higher ethanol potential ranging from 17.9 to 22.9 g/(100 g TS), in comparison with WS (Table 3). This result was mostly in line with enzymatic hydrolysis, with only two exceptions, that is, MGC and WDL. WDL contained high amount of ash (9.1 g/100 g TS), while after pretreatment, the ash content dropped down to 5.5 g/(100 g TS) and much lower mono- and oligosaccharides were detected from liquid fraction, compared to WS-P (Figure 2a,b). MGC-P yielded poorer compared to WS-P during enzymatic hydrolysis (Figure 4a,b); however, a considerable amount of saccharides was recovered from liquid fraction after MGC pretreatment, which contributed to a higher ethanol potential at 19.7 g/(100 g TS) (Table 3).

TABLE 3 Toxicity test and ethanol potential

Category	Biomass	OD max	μ (per hour)	Lag phase (hr)	Ethanol potential (g/100 g TS)
Straw	WS	9.9	0.31	1.6	17.6
	BS	13.2	0.36	3.0	21.4
	RS	12.8	0.34	2.0	12.9
	GS	13.8	0.30	2.0	18.1
	OS	12.0	0.30	3.7	17.0
Softwood	NSS	5.4	n.d.	10.5	4.9
	MSS	2.5	n.d.	10.5	4.9
Processed fiber	OW	2.9	n.d.	11.0	19.7
	BF	4.8	n.d.	11.0	1.0
	DL	13.7	0.33	3.7	12.1
	WDL	15.0	0.37	2.0	14.6
	RF	12.2	0.37	4.0	22.9
	LF	2.8	n.d.	10.0	17.9
	RC	13.3	0.33	2.5	17.5
	MGC	14.3	0.19	3.0	19.7
	RPC	14.9	0.36	5.0	4.6
	BPM	0.6	0.11	3.0	11.3
Reference	YPD	14.1	0.65	1.0	

Notes. μ is the slope intercept in exponential phase of yeast cell growth. $\mu = 0$ indicates that exponential phase was not reached. Ethanol potential values were calculated based on maximum theoretical sugar to ethanol conversion index (0.511 g ethanol/g sugar) on the basis of 100 g TS of raw biomasses. OD max, μ , and lag phase were measured in duplicates. n.d.: not detected.

The toxicity test was carried out to assess the performance of yeast in the early stage of fermentation, that is, <15 hr. Each sample was spiked with approx. 22 g/L glucose due to the fact that most liquid fractions from pretreatment contained small amount of glucose (Figure 2a). The duration of yeast cell growth lag phase was polarized among 17 studied feedstocks. Most feedstocks had a lag phase shorter than 5.0 hr, including all five straw type feedstocks, seven processed fiber feedstocks, that is, DL, WDL, RF, RC, MGC, RPC, and BPM (Table 3). Softwoods and three processed fibers (OW, BF, and LF) showed substantially longer lag phase (>10.0 hr), and these feedstocks did not overcome the lag phase within the experimental period (specific growth rate [μ] = 0 per hour) (Table 3). However, short lag phase was not necessarily correlated with low toxicity levels of some feedstocks, for example, MGC and BPM demonstrated short lag phase at 3.0 hr, but the growth rate values were significantly lower than other feedstocks with short lag phase (<5.0 hr), 0.19 and 0.11 per hour, respectively (Table 3).

3.7 | Key processing indicators

To assess the processing feasibility of these alternative feedstocks, important factors including price, logistics, and availability were taken into account. These factors were presented together with ethanol potential and fermentability in Table 4. Logistic feasibility describes the difficulty level during biomass handling and is vital from the supply chain point of view (Table 4). For example, the biomasses (DL, WDL, RF, and LF) with high water content or in a slurry form are less favorable compared to baled biomasses (e.g., straw types) in logistics (Table 4). Availability indicates the available quantity of a feedstock type within a certain distance radius from a biorefinery (Table 4). The availability of each feedstock was estimated by the production capacity of each biomass nearby Maabjerg Energy Center, Denmark. Production variations of major agricultural crops in Denmark can be found on the Statistics Denmark website (www.dst.dk/en). In this study, due to the market price fluctuation, the local farmers choose more profitable crop types to cultivate, which leads to an unstable feedstock flow of a certain type to biorefinery. Thereby, flexibility on the feedstock side is crucial for a biorefinery to ensure low feedstock prices and a stable feedstock supply.

4 | DISCUSSION

4.1 | Variations in cell wall composition and structure

The major cell wall carbohydrate components varied largely in relative quantity among the studied 16 alternative

TABLE 4 Key processing indicators comparatively scaled in six levels

Biomass	Price estimate €/ton TS	Logistics	Availability	Ethanol potential	Fermentability
WS	90	+++	+++	+	++
BS	90	+++	+++	++	++
RS	90	+++	++	–	++
GS	90	+++	+	+	++
OS	80	+++	+	+	++
NSS	0	++	+	--	---
MSS	0	++	+	--	---
OW	140	+	–	++	---
BF	–70 ^a	+++	+	---	---
DL	0	–	++	–	++
WDL	0	--	++ ^b	–	++
RF	135	+	– ^c	+++	++
LF	135	+	– ^c	+	---
RC	0	++	–	+	++
MGC	40	++	+	++	++
RPC	200	++	+++	--	++
BPM	110	++	+	–	---

Notes. Prices were estimated based on local market price in Denmark (near MEC) delivered at biorefinery.

^aNegative price indicates a cost for MEC under present-day conditions. ^bNew washing procedure should be implemented. ^cUncertainties on future production.

feedstocks, as shown in Table 1. Besides quantity, the accessibility of the cell wall carbohydrates is important when looking at real-life processing scenarios. In this study, CoMPP results provided information on the relative quantity and accessibility of hemicellulose polysaccharides, as an approach to further characterize these alternative feedstocks and to understand their different responses to hydrothermal pretreatment.

For straw type feedstocks, arabinoxylan is the major hemicellulose polysaccharides in the cell walls, while β -(1 \rightarrow 3) (1 \rightarrow 4)-D-glucan is the second most abundant hemicellulose polysaccharide (Scheller & Ulvskov, 2010). Xylans in the secondary walls are often acetylated. Hydrothermal pretreatment is known to solubilize acetyl groups from xylan backbones (Kabel, Bos, Zeevalking, Voragen, & Schols, 2007), hence to enhance pretreatment by lowering the pH. Different from the straw feedstocks, the softwoods contain galactoglucomannan as the primary hemicellulose fraction, as well as minor fraction of arabinoglucoronoxylan (Scheller & Ulvskov, 2010). Xylans in softwoods are less acetylated compared to straw type, but glucopyranuronosyl substituents are often present in softwoods to stabilize xylans (Pereira, Silveira, Dupree, & Skaf, 2017). Cleavage of glucopyranuronoxyl (pK_a 3.21) units and acetyl (pK_a 4.75) groups in softwoods during pretreatment contributed to lower pH values in liquid fraction

of softwood feedstocks compared to straw type feedstocks (Supporting Information Figure S1). Hydrothermal pretreatment removed a large part of the hemicelluloses (Table 1) and left these softwoods glucan-rich. Processed fiber feedstocks showed the largest variations in carbohydrate quantity and accessibility regardless of hydrothermal pretreatment. Among the 10 different feedstocks in this category, OW, WDL, and RC are the three most identical feedstocks to WS. Interestingly, the accessibility of major hemicellulose polysaccharides of RC and MGC was more improved during hydrothermal pretreatment, compared to all the other feedstocks (Figure 1).

The ash content also has an impact on processing efficiency. Silica is the principal component in ash, which causes a relative lower efficiency during thermochemical pretreatment and enzymatic hydrolysis (Khaleghian, Molaverdi, & Karimi, 2017). Furthermore, deposition of silica or silicates is harmful to the bioreactors (Jenkins, Baxter, Miles, & Miles, 1998). BF, DL, and MGC contained the highest ash contents, namely, 29.7, 15.1, and 13.7 g/(100 g TS), among all 17 feedstocks in this study (Table 1). After hydrothermal pretreatment, the ash levels remained high (Table 1, Supporting Information Table S1). A washing step on DL biomass could remove 40% of its ash content (WDL-Table 1); however, it led to technical complexity as well as higher water consumption during processing.

4.2 | WRV-biomass recalcitrance indicator

The multifactors contributing to certain level of resistance of biomass to thermochemical and enzymatic deconstruction are collectively known as biomass recalcitrance (Himmel et al., 2007). More specifically to this study, the natural traits of these biomasses caused varied sugar conversion yield via hydrothermal pretreatment and enzymatic hydrolysis. The changes of major cell wall components could be measured in numbers, but the structural changes introduced by processing could not. Efficient hydrothermal pretreatment does not necessarily need to remove hemicellulose completely to improve the accessibility of cellulose (Ishizawa et al., 2009). Instead, partial hemicellulose removal and lignin relocation are sufficient to enhance cellulose convertibility.

In order to measure the relative recalcitrance level of these biomasses and to assess the co-processing possibility with WS biomass, WRV was examined in correlation with glucan yield (Figure 4a,b). WRV measures the mass of water, which is closely associated with biomass, and it was recently demonstrated that increased pretreatment severity on a softwood type correlated with higher WRVs, and better glucan conversion (Weiss et al., 2017). In other words, low WRVs on pretreated biomasses indicate insufficient pretreatment for certain biomasses. WRV can provide a simple method to measure biomass recalcitrance indirectly, hence to evaluate the potential of biomasses in existing platforms.

To notice, this study indicates that the WRV cannot be applied as a sole prediction method for enzymatic hydrolysis yield of diverse biomasses. Because it is technically difficult to uniform the particle sizes due to the varied textures of biomasses, and some biomasses contain considerable amount of water in their original form. The presence of an underlying mechanism of low enzymatic convertibility of RS-P is confirmed by the WRV results, but as a method of measuring recalcitrance it should be complemented.

4.3 | Toxicity of pretreated alternative feedstocks

According to the toxicant analysis results, the liquid fractions of all pretreated alternative feedstocks did not contain furfural or 5-hydroxymethyl furfural, and acetate concentrations ranging from 0 to 1.6 g/L. However, the acetate concentrations of all liquid fractions were lower than the reported minimum inhibitory concentration of 6 g/L (Narendranath, Thomas, & Ingledew, 2001). Low growth rates of yeast can also be caused by limitations of some key nutrients, for example, nitrogen sources, trace

elements, or other supplements in the media (Hahn-Hägerdal et al., 2005). There are many factors contributing to yeast toxicity (Palmqvist & Hahn-Hägerdal, 2000; Palmqvist, Grage, Meinander, & Hahn-Hägerdal, 1999). This toxicity test cannot unveil the mechanisms of inhibition, but rather demonstrate combined toxicity levels of different biomasses within early stage fermentation.

To note, the toxicity level of alternative feedstocks cannot be translated directly into guidelines in biomass assessment for a bioethanol refinery, since, for example, a washing step after pretreatment may effectively remove toxicants from the solids. Furthermore, yeast adaptation during yeast propagation can counteract a part of the inhibition (Tomás-Pejó & Olsson, 2015).

4.4 | Co-processing assessment of alternative feedstocks

On the basis of this study, some feedstock types with poor ethanol potential and fermentability should be ruled out from WS-based processing, namely, NSS, MSS, OW, BF, LF, and BMP (Table 4). The ones with similar or better ethanol potential and fermentability to WS, including BS, GS, OS, RF, RC, and MGC, are considered as compatible feedstocks (Table 4). Taking price, logistics, and availability into account, BS is an even better feedstock overall in comparison with WS; while GS, OS, RC, and MGC with poor availability could be considered as supplementary feedstocks, biomass like RF, though demonstrating extremely promising ethanol potential, still has a too high estimated price and relatively demanding logistics, upon the current infrastructure, compared to, for example, WS (Table 4). To note, the experimental results from this study were performed only in laboratory scale and may require upscaled testing to validate the findings for large-scale applications. Likewise, the market price and availability are key processing indicators that vary from year to year, which will influence the feedstock portfolio directly. Biomass feedstock flexibility can be achieved for a range of biomasses, enabling more economic viable biorefineries.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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